LIVE ATTENUATED VACCINE AGAINST PORCINE PLEUROPNEUMONIA.

Field of the invention

The present invention relates to a method for obtaining an immunogenic, non haemolytic strain of *Actinobacillus pleuropneumoniae*, suitable to prepare a live attenuated vaccine against porcine pleuropneumonia.

Background of the invention

Actinobacillus pleuropneumonia (thereafter "App") is a Gram-negative bacteria which causes porcine pleuropneumonia, a worldwide distributed infectious disease responsible of important Economic losses in the swine industry.

The App most important virulence factors are extracellular proteins namely: Apx exotoxins. These exotoxins belong to the pore-forming RTX toxins family, widely spread among pathogenic gram-negative bacteria. The main exotoxins in App are: ApxI, ApxII, ApxIII and ApxIV.

Exotoxins ApxI and ApxII are haemolytic and cytolytic. ApxI shows a strong haemolytic and cytolytic activity and ApxII shows a weak haemolytic and a moderate cytolytic activity.

Although all, so far screened, serotypes are able to produce ApxIV, there is a characteristic serotype distribution for the expression of the rest of the Apx exotoxins. Serotypes 1, 5, 9 and 11 produce exotoxins ApxI and ApxII; Serotype 10 only produces ApxI; Serotypes 7 and 12 produce only ApxII and serotypes 2, 3, 4, 6, and 8 produce ApxII and ApxIII.

The genes corresponding to exotoxins ApxI and ApxII are organized as operons. The operon of of the ApxI exotoxin contains 4 genes: *apx*IC, *apx*IA, *apx*IB and *apx*ID. The gene of *apx*IA codes for the ApxI exotoxin itself. The gene *apx*IC codes for an activator protein (acylase) which introduces a post-translational modification (acylation) in the Apx, which allows the ApxI to acquire an active conformation, making it able for the interaction with the host specific cell receptors. The *apx*IB and *apx*ID genes code two membrane proteins which secrete the mature ApxI exotoxin to the external medium.

The ApxII operon contains only the gene A (*apx*IIA) and gene C (*apx*IIC) which code, respectively, for ApxII and for the acylase responsible for the ApxII to acquire an active conformation. There exists also a small fragment which shows a certain similarity with the *apx*IB gene but it does not originate a functional protein. The export of the mature ApxII to the external medium is due to the action of the proteins encoded by the *apx*IB and *apx*ID genes.

The present vaccination methods do not provide a complete protection against all App serotypes.

Patent WO97/16532A1 describes the construction of a vaccine strain able to induce an immunological response in an animal. This comprises a modified microorganism which produces a partially or totally inactivated Apx toxin, due to a partial deletion. This deletion, done by induced mutagenesis of the structural gene *apx*IA and/or to the partial deletion of an *apx*IIC activator gene. It does not modify the transmembrane zone.

Patent EP810283A2 describes the construction of an App vaccine strain by modifying the *Apx*IC gene in such a way that this does not produce the activator protein in a functional form and this can not activate the toxin by acylation. It neither modifies the transmembrane zone.

Jansen et al. (Infection and Immunity 63:7-37 (1995)) described the production of App homologous recombinants by site directed mutagenesis. These mutants present the *apx*IA gene which is inactivated by insertion of the CMr gene and/or the *apx*IIA gene inactivated by insertion of the *TETr* gene.

Tascón et al. (Molecular Microbiology 14 : 207-216 (1994)) describes two App mutants. One of them has a disruption in the gene *apx*IBD and the other a disruption in the structural gene *apx*IA.

Reimer et al; (Microbial Pathogenesis 18 :197-209 (1995)) describes an App. avirulent mutant which, by chemical mutagenesis, has deletions that affect important parts of the operon *apx*IABCD. This mutant does not synthesize the ApxI toxin, but is able to synthesize the ApxII, although this is not secreted from the cell.

Strains that do not express ApxI and ApxII exotoxins can not be used

as attenuated vaccines because they do not induce protective immune responses since the ApxI and ApxII exotoxins are one of the most important virulence determinants of App.

Prideaux ((The 16th International Pig Veterinary Society Congress, Melbourne (Australia) 17-20th September 2000, pag.439-442)) describes a vaccine prepared from a strain with an inactivated *apx*IIC gene that secretes and express a non-activated *Apx*II toxin unable therefore to attach to the target cells.

So, the live attenuated vaccines described in the previous background of the invention, based on App strains without haemolytic capability, are less immunoprotective because they have suffered modifications in their structure that do not allow them to attach to the membrane receptor of the target cells. Furthermore these can not generate antibodies against *Apx*I and/or *Apx*II toxins , since these are not secreted by the cell. Frey et al. Gene (142:97-102 (1994)) describe the amino-acid sequence of the ApxI exotoxine from a serotype I strain and Smiths et al.;((Infection and Immunity 59:4497-4504 (1991)) describe the amino-acid sequence of the ApxII exotoxin of a serotype 9 strain.

Summary of the invention.

The authors of the present invention have discovered a method to obtain an immunogenic and non-haemolytic App strain from an App virulent strain which has been modified in at least one segment of *apx*IA gene and optionally in a segment of the *apx*IIA gene which code a transmembrane domain of the Apx cytolytic and haemolytic exotoxins.

This strain has no haemolytic activity, but maintains unaltered its immunoprotective ability and is suitable to prepare a live attenuated vaccine against porcine pleuropneumonia.

The transmembrane domains of ApxI and ApxII exotoxins play an important role in the formation of the pore in the membrane of the target cell. Once this pore has been formed, osmotic unbalances develop which eventually

cause the lysis of the target cell.

Surprisingly it has been found, that the live attenuated vaccine prepared with these App modified strain may be administered at low dosage, that contains ApxI and ApxII toxins without haemolytic activity and that contains all the antigenes immunologically necessary to obtain a strong immunogenic response.

The object of the present invention is to develop a method to obtain an immunogenic and non-haemolytic App strain from a virulent App. strain modified in at least one segment of *apx*IA gene and optionally in a segment of *apx*IIA gene which codes a transmembrane domain of the haemolytic and cytolytic Apx exotoxins.

Furthermore, another aspect of the present invention are the strains to be obtained using the methods object of the present invention and the vaccines prepared therefrom.

In a third aspect the invention aims at the App strains deposited in the Colección Española de Cultivos Tipo with the registration numbers: CECT 5985 and CECT 5994.

Description of the figures.

Figure 1.

Fig. 1 shows an alignment performed with the ClustalX program (Thompson et al; Nucleic acid Research 24:4876-4882 (1997)) between the amino-acid sequences of ApxI coming from a serotype 1 strain ((Frey et al; Gene 142: 97-102 (1994)) and ApxII from serotype 9 ((Smits et al.; Infection & Immun. 59:4497-4504 (1991)). In this figure only the sequence contained between amino-acids 1 to 594 of ApxI and 1 to 590 of ApxII have been enclosed. On the alignment the following regions are framed : H1 (amino-acids 233 to 253), H2 (amino-acids 296 to 315) and H3 (amino-acids 369 to 405). These regions correspond respectively to the three transmembrane domains present in both Apx.

Figure 2.

Figure 2 shows a scheme with the steps followed to obtain the hybrid plasmids pApxIΔH2 and pApxIIΔH2. Firstly, the pGP1 plasmid was obtained by digestion of plasmid pGP704 ((Miller and Mekalanos; J. Bacteriol. 170: 2575-2583 (1988)) with restriction enzymes *Eco*RI and *Bgl*II and subsequent ligation of the oligonucleotides pGP5′ and pGP3′ . This plasmid contains the vegetative origin of replication of plasmid R6K (OriR6K) and the origin of transference by conjugation of plasmid RP4 (OriTP4) and of the gene of resistance to ampicilin *(Bla)*. Plasmid pGP1 was digested with the restriction enzyme PstI and ligated with a fragment PstI carrier of a gene of resistance to Kanamicin (*Km*^{r} or *Kan*) which comes from plasmid pUC4K to originate plasmid pGP2. To this plasmid, previously dephosphorylated and digested with restriction enzyme EcoRI, three DNA fragments amplified by PCR were inserted namely: the promoter ptac, the coding region of the fusion at atpE/GFPUV (ribosome binding region where the *E. coli atp*E with the U.V. variant of the green fluorescence protein) and the transcription rrnB terminator. The resulting plasmid was named pGP3. In the plasmid, the flanking regions 5′ and 3′ (amplified by PCR) that code for the second transmembrane helix of genes *apx*I and *apx*II were inserted to form plasmids pApx1ΔH2 and pApxII Δ H2 respectively.

Figure 3.

Figure 3 is divided in three panels: Panel A shows the restriction maps in kilobases (kb) and the distribution of the genes in the operon *apx*I from the genome of App. In light gray, the *apx*IA gene is depicted, being the target of the different recombination events, and in dark gray the adjacent genes *apx*IC, *apx*IB and *apx*ID. The different genes or regions of plasmid pApxIΔH2 are drawn using skewed bars. The coding fragments of the transmembrane helices (H1, H2 and H3) of apxIA are highlighted in Black. The names and some detailed structures in figure 2 plasmids have been simplified. Thus gfpUV comprises the ptac promoter and the atpE/GFPUV fusion; OriV indicates the

vegetative origin of replication of R6K and OriT the origin of transference by conjugation of RP4. In (1) and (3) both are shown the restriction map obtained with enzyme *Xho*I and the distribution of the genes of operon *Apx*I of the App genome are shown. In (2) the restriction map of the same operon after the insertion of plasmid pApxIΔH2 in the App genome is shown. This insertion occurs by a unique homologous recombination event between flanking regions 5′ of H2 placed in plasmid pApxIΔH2 and the App genome respectively.

Panel B of figure 3 shows the results of the hybridisation of an *Apx*1 gene probe with a southern transference of the digested genomic DNA digested with *Xho*I and obtained from the harvests of: 1)HP816 Nl^r ; 2) recombinant obtained by insertion of plasmid pApxIΔH2 in the App genome through a unique homologous recombination event between the flanking regions 5′ of H2 (HP816R1); 3) recombinant obtained from HP816R1 which recovers the same phenotype of the parent strain HP816 Nl^r and 4) recombinant obtained from HP816R1 which recovers the same phenotype of the parent strain HP816 Nl^r with the exception that it shows the same haemolytic activity than the HP816R1 (AppApxIH2$^-$).

Panel C shows the aspect of the colonies seeded from the same cultures described in panel B (1, 2, 3 and 4) together with another colony coming from a serotype 7 App culture (5). The colonies were seeded on Columbia blood agar plates supplemented with 0.004% NAD (CA)or in TSYN plates supplemented with 25 μg/mL kanamicin (TS). The presence of large haemolytic halos surrounding the colonies of cultures 1 and 3 and of small haemolytic halos surrounding the colonies in cultures 2, 4 and 5 can be seen in CA. Also the absence of growth in TS of cultures 1, 3, 4 and 5.

Figure 4

Figure 4 is divided in 3 panels: Panel A shows the restriction maps (in kb) and the distribution of the genes in operon *apx*II located in the App genome. In light grey the *apx*IIA gene is depicted. This is the target of the different recombination events. The adjacent genes *apx*IIC, *apx*IIB are depicted in dark grey ; The different genes or regions of plasmid pApxIIΔH2

are drawn using skewed bars. The coding fragments of the transmembrane helices (H1, H2 and H3) of apxIIA are highlighted in black. The restriction map obtained with the enzyme *Eco*RI and the distribution of the ApxII operon genes of the App genome are shown in (1) and (3). The restriction map of the same operon after the insertion of pApxIIΔH2 plasmid in (2) is depicted. This insertion is done through a unique homologous recombination event between the 3′ flanking regions of H2 located in plasmid pApxIIΔH2 and the App genome. In (4) the restriction map of the operon is depicted after the resolution of the plasmid inserted in (2) after a second recombination through the 5′ flanking regions of H2 located in the App genome.

Panel B shows the hybridisation results of a probe of gene *apx*IIA on a Southern transfer of the genomic DNAs digested with *Eco*RI and obtained from the following cultures of: 1) HP8816 Nl^r 2) Recombinant obtained by insertion of plasmid pApxIIΔH2 in the genome of App through a unique homologous recombination event between the 5′ flanking regions of H2 (HP816R2); 3)recombinant obtained from HP816R2 which recovers the same phenotype of the parent strain HP816 Nl^r ; 4) recombinant obtained from HP816R2 which recovers the same phenotype of the parent strain HP816 Nl^r with the exception that it shows the same haemolytic activity than HP816R2 (AppApxI/IIH2$^-$).

Panel C shows the aspect of the colonies seeded from the same cultures described in panel B (1, 2, 3 and 4). The colonies were seeded in Columbia blood agar plates supplemented with NAD (CA), or in TSYN plates supplemented with 25 μ/mL of kanamycin (TS). The presence of small haemolytic halos surrounding he colonies of cultures 1 and 3 and the absence of haemolytic halos surrounding the colonies of cultures 2 and 4 can be seen. See also, in TS, the absence of growth of cultures 1, 3 and 4.

Figure 5

Figure 5 shows three graphs with the growth curves, represented (dark symbols) from the absorbance values, at 600 nm, of the different cultures, at a one hour intervals (left y-axe). Simultaneously, several samples were taken

from the supernatants of the cultures in the same time intervals. These samples were kept at 0 C until these were diluted 1/50 in carbonate buffer (pH 9.6). These were then placed in microwells to quantify the presence of ApxI and ApxII by ELISA using a monoclonal antibody specific for each Apx. From the absorbance values, at 405 nm, of each tested sample (right y-axis), the curves were drawn representing the accumulation of each one of the Apxs along the time (light symbols). In (A) a HP816 Nl^r culture –triangles- and an AppApxIH2$^-$ culture – circles-; the light symbols show the ApxI accumulation . In (B) a culture of HP816 Nl^r -triangles- and an AppApxI/IIH2$^-$ culture – circles- the light symbols show the accumulation of ApxII. In (C), a culture of HP816RI –triangles- and, , a culture of HP816R2 culture –circles- show the accumulation of ApxI and the light circles show the accumulation of ApxII.

Figure 6

Panel (A) shows a Coomassie blue staining of a denaturing electrophoresis in polyacrilamide gel with samples of the supernatants of cultures taken at 5 hours from 1) HP816 Nl^r; 2) AppApxIH2$^-$; 3) a control obtained from an App serotype 4 (the App serotype 4 produces and secretes the ApxII of 105 kD and ApxIII of 115 kD but not the ApxI); and M) marker of a molecular mass (the relevant bands of 110 and 120 kD are indicated). In (B) we can observe a Western-blot of a gel with identical samples to those analysed in gel (A) , detected by means of a monoclonal antibody specific for the ApxI. In (C) we can observe a, Western-blot of a gel with identical samples to those of gel (A) with the exception of track 2, which contains a sample of the supernatant of AppApxI/IIH2$^-$ culture. No picture of the gel is enclosed since the band distribution is identical to that which appears in gel (A). This transfer was revealed using a specific monoclonal antibody for ApxII. Observe that the band of 105 kD of track 3 appears detected only in (C).

Detailed description of the invention.

The invention refers to a method to obtain an immunogenic and

non- haemolytic strain of *Actinobacillus pleuropneumoniae* from a virulent App , characterized by the following stages:

- The transmembrane domains of the Apx haemolytic and cytolytic exotoxins are determined.

- At least one fragment of gene *apx*IA is modified and optionally a segment of the *apx*IIA gene which codes a transmembrane domain of the Apx haemolytic and cytolytic Apx exotoxins.

The term immunogenic means that the App strain obtained with the method of the present invention maintains unaltered its immunoprotective ability, this means that it contains all antigens immunologically necessary to obtain a high immunogenic response in the host.

The term non-haemolytic means that the App strain, obtained with the method of the present invention, has no haemolytic activity, being an avirulent strain.

The choice of an App virulent strain, obtained from infected animals which suffer the disease, is done according to the usual methods known by those skilled in the art know.

The first step consists in the identification of the transmembrane domains of the App haemolytic and cytolytic Apx exotoxins using the TransMem programs (Aloy et al; Comp. Appl. Biosc. **13**: 213-234 (1997)) or Helixmem (Eisenbeg et al.; J. Mol.Biol. **179**: 125-142 (1984)) which analyses the amino-acid sequences of the haemolytic and cytolytic Apx exotoxins, as described for E. coli in Ludwig et al.; Mol. Gene. Genet. **226**: 198-208 (1991).

The second stage consists in modifying at least one segment of the *apx*IA gene and optionally a segment of *apx*IIA gene which code a transmembrane domain of the Apx haemolytic and cytolytic exotoxins.

The term "modify" refers to the modification of a gene either by using DNA recombinant conventional techniques which include : the substitution of one or several nucleotides, the insertion of one or several nucleotides, the partial or total deletion of a gene, or through the disruption by chemically or radiation induced mutagenesis.

In a preferred realization, the modification is performed by deletion in, at

least, one segment of *apx*IA gene and optionally in a segment of *apx*IIA gene, which code a transmembrane domain of the haemolytic and cytolytic Apx exotoxins.

The transmembrane domains, present in the *apx*IA and in *apx*IIA genes of the haemolytic and cytolytic exotoxins, were detected using the Transmem and Helixmem programmes above mentioned. The prediction performed on the amino-acid sequences of the haemolytic and cytolytic exotoxins ApxI and ApxII indicate that the transmembrane domains, also named transmembranes , are found located in the following zones of the sequence of the exotoxins:

- First transmembrane domain H1: between amino-acids 233 and 253 corresponding to the nucleotides 699 to 759 from *apx*I.
- Second transmembrane domain H2: between amino-acids 296 and 315, corresponding to nucleotides 888 to 945 from a*px*I
- Third transmembrane domain H3: between amino-acids 369 to 405, corresponding to nucleotides 1107 to 1215 from *apx*I

In a preferred realization, the modification is carried out by means of a deletion in the segment of gene *apx*IA which codes the second transmembrane domain of the exotoxin ApxI of App.

The modification is carried out, preferably, by deletion of the nucleotides 885 to 944 of the *apx*IA gene which code the second transmembrane domain of the App *Apx*I exotoxin.

Another preferred realization, of the method object of the present invention, furthermore introduces an additional deletion in the segment of *apx*IIA gene which codes the second transmembrane domain of the ApxII exotoxin of App. Preferably a deletion of nucleotides 885 to 944 of gene *apx*IIA, which code the second transmembane domain of the App *Apx*II exotoxin.

In the preferred realization form of the present invention, which will be described in detail in the section "examples", the achievement of an immunogenic non-haemolytic App strain has been performed using a process comprising the following steps:

A – Selection of an App virulent strain.

B – Prediction of the alfa helices of the transmembrane domain of the ApxI and ApxII proteins in order to design a nucleotide construction which allows the deletion in the second transmembrane domain of both proteins without affecting the folding process and the capacity of the resulting haemolysins to interact with the membrane specific receptors.

C – Construction of a cloning vector able of integrating in the App genome and which contains marker genes which allow the monitoring , in an efficient manner, the integration of such a vector.

C1 – Construction of the hybrid plasmid pGP3 which has an origin of replication RK6, the RP4 origin of transfer and a gene of resistance to kanamycin. Furthermore, the gene of the fluorescent protein GPVUV (thereafter GFP)was enclosed, under the control of promoter ptac and terminator rrnB. The multiple cloning site was also modified to ease the later insertion of the DNA sequences.

C.2 . Construction of a hybrid cloning vector which contains the 5′and 3′ flanking sequences of the second transmembane helix, specified by the apxIA gene. Therefore, the hybrid plasmid pApxIΔH2 was constructed in order to select and clone such fragments adjacent to the 5′ and 3′ ends of the segment that codes the second transmembrane helix in the *apx*IA gene. This plasmid was used as the final vector for the transformation of App.

C.3. Construction of a hybrid clonation vector which contains the 5′and 3′ flanking sequences of the second transmembrane helix specified by the *apx*IIA gene. Therefore the hybrid plasmid pApxIIΔH2 was constructed in order to choose and clone such fragments adjacent to the 5′ and 3′ ends of the segment that codes the second transmembrane helix in the *apx*IIA gene. This plasmid was used as a final vector for the transformation of App.

D. Construction of the recombinant bacteria which have resolved the hybrid plasmid inserted in the genome.

D.1. Construction of the App recombinant strain named HP816R1, which incorporates the hybrid plasmid pApxIΔH2 in the App genome as a result of a unique homologous recombination event between such plasmid and the genome of App HP816 Nl^r which is a strain resistant to nalidixic acid obtained from a spontaneous mutant of the App HP816 strain.

D.2 Construction of the strain AppApxIH2$^-$ using a procedure that allows , once the recombinant bacteria obtained in step 1 have been identified, to resolve the recombinant vector integrated in the genome through a second homologous recombination event, as well as to detect and isolate the bacteria that, due to this second recombination, have achieved the partial deletion in the *apx*IA gene.

D.3 Construction of the App HP816R2 recombinant strain, which incorporates the hybrid plasmid pApxIIΔH2 in the genome of AppApxIH2$^-$ as a result of a unique homologous recombination event between such plasmid and the genome of AppApxIH2$^-$.

D. 4 Construction of the AppApxI/IIH2$^-$ strain using a procedure that allows , once the identified recombinant bacteria have been obtained in step D.3, to resolve the recombinant vector integrated in the genome by means of a second homologous recombination event., as well as to detect and isolate the bacteria that , due to the second recombination , have acquired the partial deletion in *apx*IIA gene.

In the present invention the obtained mutant AppApxIH2$^-$ is identical to the original wild type App strain, except for the deletion of nucleotides 885 to 944 (both inclusive) of the coding sequence of gene *apx*IA which corresponds with that absence of the amino-acids 296 to 315 (both inclusive) in the produced ApxI .

In he present invention the obtained mutant AppApxI/IIH2$^-$ is identical to strain AppApxIH2$^-$ except by the deletion of nucleotides 885 to 944 (both inclusive) of the coding sequence of gene *apx*IIA which corresponds with the absence of amino-acids 296 to 315 (both inclusive) in the ApxII produced.

In the preferred realization of the present invention the only modifications which are produced in the App genome are the deletion of 60 base pairs within the coding sequences of genes *apx*IA and *apx*IIA and the substitution of the same by restriction targets, in this case the corresponding to the enzymes *Xho*II and *Eco*RI respectively. No additional insertions of sequences coming from plasmidic DNA are produced (as for instance the gene of resistance to the kanamicin) in the App genome. This allows the obtained strain to be modified in the same gene o in another target gene following

exactly the same strategy which was used to carry out the first modification. On the other hand, it avoids that the resulting strain be resistant to multiple antibiotics, which is a desirable characteristic in a strain to be used as live vaccine.

The restriction target used in the present invention is not critical. Although a construction could be used that introduced no restriction target, its use is preferable in order to have an additional mechanism to detect the desired recombinant clones and to be able to perform a follow up of the stability of the strain, or strains obtained. Therefore any target can be used proving that it does not introduce a stop codon of the protein synthesis and that gives rise to restriction fragments analysable by electrophoresis (i.e. more than 100 base pairs) with flanking restriction fragments which might exist previously in the App genome.

The invention refers also to an App strain obtained according to the above described method.

Another object of the invention is an App strain characterized because it has a deletion in nucleotides 885 to 944 of the *apx*IA gene, that code the second transmembrane of the ApxI exotoxin, deposited in the Colección Española de Cultivos Tipo (Spanish Collection of Type Cultures) with the registration number CECT 5985, according to the Treaty of Budapest of 28th April 1977, or a mutant thereof.

Another object of the invention is an App strain characterized by having a deletion of nucleotides 885 to 944 of the *apx*IA gene that code the second transmembrane of the ApxI exotoxin and besides a deletion of nucleotides 885 to 944 of *apx*IIA gene that code the second transmembrane of the ApxII exotoxin deposited in the Spanish Collection of Type Cultures with the registration number CECT 5994, according to the Budapest Treaty, or a mutant thereof.

The invention also refers to the vaccines for the protection of the animals against porcine pleuropneumoniae. These vaccines may be prepared according to the usual methods known by those skilled in the art.

These vaccines comprise an immunologically effective amount of bacteria of a live App attenuated strain, obtained according to the method

described in the present invention. Immunologically effective means that the amount of bacteria administered for the vaccination is sufficient to induce in the host an effective immunological response against an infection caused by virulent forms of App.

The dosage to be used will depend on the age and weight of the animal to be vaccinated and the administration form.

The vaccine may contain whichever bacterial dose sufficient to induce an immune response. The suitable dosage is comprised in the range 10^3 and 10^{10}.

The vaccine may contain furthermore a pharmaceutically acceptable excipient. This excipient may be as simple as water, but it may also comprise the culture fluid in which the bacteria are grown or a solution with a physiological concentration of salt.

Another example of pharmaceutically acceptable excipients , useful in the present invention enclose stabilizers , carbohydrates (i.e.: glucose, sacharose, manitol, sorbitol) and buffers (i.e.: phosphate buffers).

Optionally other adjuvant components may be added to the vaccine. These adjuvants are non-specific stimulants of the immune system which augment the immune response of the host against the pathogenic invader. Examples of adjuvants are: Vitamin E and vegetal oil.

The vaccine may be administered, to animals, by intranasal, intradermal, subcutaneous, spray or intramuscular routes.

The industrial application of the invention is easily deduced from the description. It is worth mentioning that porcine pleuropneumonia is a worldwide infectious respiratory disease responsible of severe economic losses to the porcine industry and that the method of the present invention to obtain immunogenic, non-haemolytic App strains allows the preparation of efficacious vaccines to fight porcine pleuropneumonia.

The examples that follow are described in order to provide, to the skilled in the art, a sufficiently comprehensive and complete explanation of the present invention, but these must not be considered as limitation to the essential aspects of the same as described in the previous section of this description.

Example

The techniques and DNA recombinant methods applied as follows, are described in detail in Sambrook and Russell ((In Molecular cloning 3rd Ed. Cold Spring Harbor Laboratory Press, Cold spring Harbor New York (2001) and Ausubel et al; *Curent Protocols in Molecular Biology*, John Wiley and Sons, Inc. (1998)). All PCR products were previously cloned in a pBE plasmid before being digested with restriction enzymes. This plasmid is a derivative of pBluescript SK2 (Stratagene) vector and presents the multiple cloning site substituted by a small nucleotidic sequence which specifies only the target of the restriction enzyme *Eco*RV.

The E. coli XL1-blue strain (Stratagene) has been used as a host for hybrid vectors based on plasmids pUC118 or pBluescript SK. The E. coli S17-1 λ pir strain ((Simon et al; Biotechnology 1:784-791 (1983)) has been used as a host of the hybrid vectors based in plasmid pGP704.

All the oligonucleotide sequences described hereunder are written in the sense 5′ to 3′unless it is explicitly otherwise indicated. In all PCR reactions, the Deep Vent thermopolymerase (New England Biolabs), which has test correcting activity, was used.

A. Selection of a virulent App strain.

The HP816 strain, which corresponds to a natural type 1 serotype of App. belonging to Laboratorios Hipra S.A. (Amer – Girona –Spain), was chosen as a wild type App strain.

Strain HP816N1^{r} is a strain resistant to nalidixic acid obtained from a spontaneous mutant of the wild type HP816.

B. Identification of the transmembrane domain of exotoxins ApxI and ApxII.

The three transmembrane domain which adopt an α- helix structure, were determined by means of the use of programmes TransMem ((Aloy *et al*; Comp. Appl. Biosc. 13:213-234 (1997)) and Helixmem ((Eisenbeg *et al*; J. Mol. Biol.179: 125-142 (1984)) as described for E. coli ((Ludwig *et al*; Mol.

Gene. Genet. 226:198-208 (1991)) applied to the amino-acid sequence of the ApxI coming from a serotype 1 strain ((Frey *et al*; Gene **142:** 97-102 (1994)) and the ApxII of a type 9 serotype ((Smits *et al*; Infection and Immunity 59:4497-4504 (1991)). These programmes detected three regions which could act as transmembrane helices in both proteins (Fig 1): the first transmembrane is located between amino-acids 233 and 253 (H1); The second transmembrane is located between amino-acids 296 and 315 (H2) and the third transmembrane between amino-acids 369 and 405 (H3) all of them from ApxI.

C. Construction of hybrid cloning vectors which are able to integrate in the App genome.

In figure 2 we can see an outline with the maps of the plasmids which are described in this section.

C.1.- Construction of the recombinant plasmid pGP3.

Plasmid pGP704 ((Miller and Mekalanos; J. Bact. **170**:2575-2583 (1988)) was cut simultaneously with restriction enzymes *Bgl*II and *Eco*RI. Using electrophoresis in agarose gel a 3.7 kb DNA fragment was isolated. This fragment incubated in a ligation reaction together with oligonucleotides pGP5′ (GAT CGA ATT CAG GAT ATC ACA GAT CT) (SEQ ID NO 1) and pGP3′ (ATT TAG ATC TGT GAT ATC GTG AAT TC) (SEQ ID NO 2). The obtained recombinant plasmid was named pGP1.

The pGP1 plasmid was digested with the restriction enzyme *PstI*. Using electrophoresis in an agarose gel a 3.12 kb DNA fragment was isolated. This fragment was ligated to another fragment of 1.2 kb obtained by digestion of the pUC4K plasmid (Pharmacia) with the restriction enzyme *Pst*I. The so obtained recombinant plasmid was named pGP2.

Using plasmid pMAL-p2 (New England Biolabs) the sequences corresponding to promoter ptac were amplified by PCR using the ptac5′oligonucleotide primers (GAA TTC AAT GCT TCT GGC GTC AG)

(SEQ ID NO 3) and ptac3′ (GGT ACC GGA TGA GAT AAG ATT TTC) (SEQ ID NO 4) which enclose respectively the restriction targets *Eco*RI and *Kpn*I in its 5′ends. Also from pMAL-p2 plasmid, by PCR the sequences corresponding to the rho- independent terminator of operon *rrn*B were amplified using the primer oligonucleotides *rrn*B5′ (GGT ACC GGA TGA GAT AAG ATT TTC) (SEQ ID NO 5) and *rrn*B3′ (GAA TTC AAG AGT TTG TAG AAA CGC) (SEQ ID NO 6) which enclose respectively the restriction targets *Kpn*I and *Eco*RI in their 5′ ends. The size of the DNA amplified fragment comprises 278 base pairs (bp).

With the plasmid pAG408 ((Suarez *et al*; Gene **196:** 69-74 (1997)) a fusion of the gene of the GFPUV protein with the region that links to the ribosome of the *atp*E gene was amplified using the primer oligonucleotides GFP5′ (GGT ACC TAA TTT ACC AAC ACT AC) (SEQ ID NO 7) and GFP3′ (GGT ACC TTA TTT GTA GAG CTC ATC) (SEQ ID NO 8) which encloses the restriction target *Kpn*I in its 5′ends. The amplified fragment has a size of 830 bp.

The two first fragments (promoter ptac and terminator rrnB) were digested with the restriction enzymes *Kpn*I and *Eco*RI whereas that the third (fusion atpE-GFPUV) was digested with the restriction enzyme *Kpn*I. The three fragments obtained in this way were then ligated with the plasmid pGP2 which was previously dephosphorylated and cut with the restriction enzyme *Eco*RI. . Among the different recombinant plasmids obtained one, which was the carrier of the three fragments positioned according to Figure 2, was chosen. The colonies that carried this plasmid showed an intense fluorescence when exposed to ultraviolet light. The hybrid plasmid so obtained was named pGP3.

C.2 – Construction of the hybrid plasmid pApxIΔH2.

At this stage, the first objective was to obtain a DNA fragment contiguous to the 5′ end of the coding fragment of the second transmembrane helix of *apx*IA gene. Therefore a fragment of 897 bp was amplified by PCR from the purified geneomic DNA of the App strain HP816 using as primers the

ApxIa5'oligonucleotides (GAT ATC ATG GCT AAC TCT CTC AGC TCG ATA G) (SEQ ID NO 9) and ApxIa3' (CTC GAG GCC TGC CGC CAC ACG TTG) (SEQ ID NO 10), which enclose the restriction targets *Eco*RV and *Xho*I in its respective 5'ends. The 7th base of the oligonucleotide ApxIa5' (SEQ ID NO 9) corresponds with the first base of the start codon of the translation of *Apx*Ia gene . The seventh base of oligonucleotide ApxIa3' (SEQ ID NO 10) is complementary to the 885 base of the coding sequence of gene *apx*IA, being the latter the last base before the initiation of the sequence for the second transmembrane helix.

The second objective of this phase was to obtain a DNA fragment contiguous to the 3'end of the coding segment of the second transmembrane helix of *apx*IA gene . Therefore , using PCR, a fragment of 1042 bp was amplified from purified genomic DNA of the strain App HP816 using as primers the oligonucleotides ApxIb5' (CTC GAG CCG CTT TCG TTC TTA AAT GTT GCG) (SEQ ID NO 11) and ApxIb3' (AGA TCT TCA CCG GCT TTC TGT GCA CTT TG) (SEQ ID NO 12) which include the restriction targets *Xho*I and *Bgl*II in its respective 5' ends. The 7th base of oligonucleotide ApxIb5' (SEQ ID NO 11) corresponds with the base 944 of the coding sequence of the *apx*IA gene, being this one the first base after the end of the sequence for the second transmembrane helix. The seventh base of oligonucleotide ApxIb3' (SEQ ID NO 12) is complementary to base 1975 of the coding sequence of the gene *apx*IA.

Once the two fragments previously described have were obtained, the first one was digested with the restriction enzymes *Eco*RV and *Xho*I, whereas that the second was digested with the enzymes *Xho*I and *Bgl*II. Both fragments were then ligated with vector pGP3 which was previously cut with the restriction enzymes *Eco*RV and *Bgl*II. The resulting hybrid plasmid was named pApxIΔH2.

C.3.- Construction of the hybrid plasmid pApxIIΔH2 .

The first objective, at this stage, was to obtain a DNA fragment contiguous to the 5' end of the coding segment of the second transmembrane

helix of gene *apx*IIA. Therefore, by PCR, a 871 bp fragment was amplified from the purified genomic DNA of the App strain HP816 using as primers the oligonucleotides ApxIIa5′ (GAT ATC AAA TCG TCC TTA CAA CAA GGA TTG) (SEQ ID NO 13) and ApxIIa3′ (GAA TTC ACC TGA AGC GAC TCG TTG GGC) (SEQ ID NO 14) which enclose the restriction targets *Eco*RV and *Eco*RI in its 5′ respective ends . The number 7 base of oligonucleotide ApxIIa5′ (SEQ ID NO 13) corresponds to base 27 of the coding sequence of the gene *apx*IIA. The seventh base of oligonucleotide ApxIIa3′ (SEQ ID NO 14) is complementary to the base 885 of the coding sequence of gene *apx*IIA, being this one the last base before the start of the sequence for the second transmembrane helix.

The second objective of this step, was to obtain a DNA fragment contiguous to the 3′end of the coding segment of the second transmembrane helix of *apx*IIA gene. Therefore a 952 bp fragment was amplified by PCR from the purified genomic DNA from the App strain HP816 using as primers the oligonucleotides ApxIIb5′ (GAA TTC CCT CTT TCA TTC TTA AAT GTA GC) (SEQ ID NO 15) and ApxIIb3′ (AGA TCT GCC ATC AAT AAC GGT AGT ACT TG) (SEQ ID NO 16), which enclose the restriction targets *Eco*I and *Bgl*II at its 5′ respective ends. The 7th base of oligonucleotide ApxIIb5′ (SEQ ID NO 15) matches up with the base 944 of the coding sequence of gene *apx*IIA, being the latter the first base after the end of the sequence for the second transmembrane helix. The seventh base of the oligonucleotide ApxIIb3′ (SEQ ID NO 16) is complementary to the base 1845 of the coding sequence of the *apx*IIA gene .

Once the two fragments described previously were obtained, the first one was digested with the restriction enzymes *Eco*RV and *Eco*RI, whereas that the second one was digested with the enzymes *Eco*RI and *Bgl*II. Both fragments were then ligated with the vector pGP3 previously digested with the restriction enzymes *Eco*RV and *Bgl*II. The resulting hybrid plasmid was named pApxIIΔH2.

D.- Construction of the recombinant bacteria which have resolved the hybrid plasmid inserted in the genome.

D.1. Construction of the App recombinant strain HP816R1.

The transformation of App with the hybrid plasmid pApxIΔH2 was done by conjugation from the E. coli S17-1 λ pir cells which are carriers of this plasmid. The strain HP816Nl^r was used for the transformation with the hybrid plasmid pApxIΔH2.

Before carrying out the conjugation, a culture in stationary phase was obtained for such bacteria. The culture medium TSYN (Soya Tryptic broth 30 g/L, yeast extract 6 g/L and once autoclaved supplemented with 0.004% NAD) and nalidixc acid (50 μg/mL) was used for the growth of App816Nl^r.

The culture medium LB (Tryptone 10 g/L, yeast extract 5g/L, NaCl 10g/L) that once autoclaved was supplemented with 25 μg/mL of kanamycin was used to grow E. coli S17-1 λpir. Once the stationary phase was achieved, 0.2-0.3 A_{600} units of the App culture and 0.6-0.8 A_{600} units of the E. coli culture were added to 1 mL of a 10mM solution of $MgSO_4$. Next it was centrifuged during 2 minutes at 15,000 g and the pellet so obtained was resuspended in 200 μl of a 10 mM $MgSO_4$ solution. Once the mixture of both cultures had been done, this was extended on a 2.5 cm and 0.45 μm nitrocellulose filter previously placed on a Petri dish containing TSYN medium supplemented with 15 g/L Noble agar. After incubation during 6 hours at 37 C, the filter with the conjugation was placed in a tube containing 2 mL of PBS (Na_2HPO_4 10 mM, KH_2PO_4 1mM, NaCl 137 mM, KCl 2mM pH 7.4). After vigorous shaking, the filter was removed and the cell suspension was centrifuged during 2 minutes at 15,000 g and the pellet was resuspended in 500 μL of PBS. The so obtained suspension was distributed in Petri dishes with TSYN medium supplemented with 15 g/L Noble Agar, 50 μg/mL kanamycin and 50μg/mL nalidixic acid, at a rate of 100 μL of cell suspension for each Petri dish. The resulting cultures were incubated at 37 C for 24-36 hours. With this procedure 65 colonies resistant to kanamicin and nalidixic acid, were obtained for the conjugation with the plasmid pApxIΔH2, which equals a frequency of transformation of $1.3x10^{-7}$ for each receptor cell.

Several colonies were reseeded by exhaustion of the loop in Petri dishes containing LP supplemented with 15 g/L Noble Agar, 0.004% NAD, 50 µg/mL kanamycin and 50 µg/mL nalidixic acid. All the resulting colonies exhibited distinct degrees of fluorescence when exposed to ultraviolet light, which indicated the integration of the plasmid in the App genome in a unique recombination event. As shown in figure 3, if a double recombination took place, the exconjugates would be unable to grow in a kanamycin containing medium. The presence of this antibiotic in the plates allows only the growth of those recombinants that have integrated the entire plasmid in its genome. The indicator gene GFP allows to discriminate if any of the colonies which are resistant to the kanamycin is the product of a spontaneous mutation.

Finally, the obtained recombinants were originated from a homologous recombination between a plasmid and the *apx*IA gene. This was verified by observing the haemolytic activity of the recombinants in Columbia blood agar plates supplemented with 0.004% NAD (Figure 3, panel C) The recombinants obtained with plasmid pApxIΔH2 exhibit a sharp decrease of the diameter of the haemolytic halo compared to the parent strain HP816Nl^r.

One of the recombinants obtained with the plasmid pApxIΔH2 was selected for the later passages and was referred to as HP816R1.

D.2 Construction of the strain AppApxIH2$^-$

Once the recombinants with the plasmid pApxIΔH2 integrated in the genome have been obtained, it is essential to fix the deletion in the App genome by means of a second recombination. Therefore one of the recombinants of the previous stage was submitted to serial passages in a culture medium supplemented only with nalidixic acid. A medium without kanamicin allows that, in case that a second recombination between the App genome and the integrated plasmid occurs, the resulting bacteria be viable. This second recombination event gives rise to the appearance of two different genotypes. In case that this occurs in the same segment in which the first recombination took place, the resulting genotype will be identical to the parent strain used in section D.1. If this second recombination occurs in

the segment where the first recombination did not take place, the resulting genotype will show a deletion in the fragment that codes for the second transmembrane helix of the haemolysin (Figure 3, panel A). The appearance off recombinants may be monitored in several different ways: a) disappearance of the fluorescence when the colonies are exposed with ultraviolet light ; b)sensibility to kanamicin and c) recovery of the haemolytic halo exhibited by the parental strain used in section D.1. Methods a) and b) detect both recombinant types. Method c) makes possible to distinguish those recombinants which recover the parental geneotype. This is due to the fact that the recombinants which show the deletion in the coding segment of the second transmembrane helix of the *apx*IA gene, the haemolytic activity of the corresponding parental phenotype is not re-established.

The serial passages were performed from previous passages using 1/10000 dilutions of the previous passage, with the exception of the first passage which simply consisted of a culture obtained from a colony isolated from HP816R1. The culture medium was LB supplemented with 0,004% NAD and 50 μg/mL of nalidixic acid. For each passage a volume of 10 mL of medium was used.The percentages of detected recombinants are shown in table 1

Table 1

Passage Number	% detected recombinants (a)	% detected recombinants (b)
2	0.12%	0.18%
3	0.32%	0.46%
4	7.75%	10.4%
5	18.5%	22.3%

a) percentage determined by colony counting which recovered the haemolytic halo which was exhibited by the parent strain .

b) percentage determined by counting the colonies that did not show fluorescence when exposed to ultraviolet light.

As observed in the table above, in each passage the number of bacteria that show a resolved plasmid due to a second recombination increases. The percentage of non-fluorescent colonies is only slightly higher than that of the colonies that rEcover the haemolytic activity. This fact suggests that the second recombination occurs preferably in the same DNA segment where the first recombination occurred. If the frequency for each recombinat type were of 50%, double the number of non –fluorescent colonies with respect to those that recover the haemolytic activity.

Once the culture has been sufficiently enriched in second recombinants, the purification step can be initiated. With this aim in mind, several non-fluorescent colonies were propagated in Columbia agar supplemented with NAD and LB agar supplemented with NAD, 50 μg/mL, nalidixic acid and 20 μg/mL kanamicin (LBNKm). For later studies, several colonies were chosen that did not show growth on LBNKm and that showed the same haemolytic activity that the recombinant by insertion HP816R1.

D.3 Construction of the App HP816R2 recombinant strain.

The transformation of the App with the pApxIIΔH2 was performed by conjugation from the E. coli S17-1 λpir which are the carriers of these plasmids. The strain AppApxIH2$^-$ was used for the transformation with the hybrid plasmid pApxIIΔH2. The procedures and the culture media are identical to those described in section D.1. The transformation frequency with the plasmid pApxIIΔH2 was similar to that obtained is section D.1. for plasmid pApxIΔH2.

Several colonies were reseeded by exhaustion of the loop in Petri dishes with LB supplemented with 15g/L Noble agar 0.004 % NAD, 50 μg/mL kanamicin and 50 μg/mL nalidixic acid. All resulting colonies exhibited distinct degrees of fluorescence when exposed with ultraviolet light, which indicates the integration of the plasmid in the App genome in a single recombination event. As observed in fig 4, if a double recombination occurs , the exconjugates will be unable to grow in a kanamicin containing

medium. The presence of this antibiotic in the plates allows only the growth of those recombinants which have integrated in its genome the entire plasmid. The indicator gene GFP allows to discriminate if any of the colonies, which are resistant to kanamicin, is the product of a spontaneous mutation.

Finally the obtained recombinants were originated from a homologous recombination between the plasmid and the *apx*IIA respective gene. This was proved by observing the haemolytic activity of the recombinants in Columbia agar plates supplemented with 0.004% NAD (fig 4, panel C). The recombinants obtained with the plasmid pApxIIH2 show a complete disappearance of the haemolytic halo.

One of the recombinants obtained with the plasmid pApxIIΔH2 was chosen for later passages and named HP816R2.

D.4 Construction of the AppApxI/IIH2⁻ strain.

Once the recombinants had been obtained with plasmid pApxIIΔH2 integrated in the genome it was essential to fix the deletion in the App genome by means of a second recombination. Therefore the recombinants obtained in the previous stage were submitted to serial passages in culture medium supplemented only with nalidixic acid as described in D.2. The values of the percentages of recombinants detected from the second passage, are similar to those obtained in D.2.

Once the culture is sufficiently enriched in secondary recombinants, the purification stage can proceed. With this aim in mind, several non-fluorescent colonies were multiplied in Columbia agar supplemented with NAD and LB agar supplemented with NAD, 50 µg/mL nalidixic acid and kanamicin 20 µg/mL (LBNKm). For later studies several colonies were chosen which did not show growth on LBNKm and showed the same haemolytic activity than the recombinant by insertion HP816R2.

E.- Analysis of the DNA purified from the colonies, isolated in the previous passage, to test the homogeneity of the cultures and the presence of the

deletion in genes apxIA and *apx*IIA.

The recombinants of the previous passages D.2 and D.4 were grown in 10 mL of TSYN medium supplemented with 50 µg/mL nalidixic acid until the stationary phase was reached. Later the DNA extraction of each one of them was performed.

E.1.- Analysis of the apxIH2$^-$ recombinants.

The samples of the genomic DNA corresponding to each one of the cultures of the second recombinants obtained from plasmid pApxIΔH2 were digested with the restriction enzyme *Xho*I. These digestions, together with others performed from the DNA extracted from the cultures of the strain HP816N1^r and HPB816R1 respectively , were analysed by Southern-blot using the fragment of DNA of 1927 bp coming from the digestion of plasmid pΔApx1H2$^-$ with the restriction enzymes *Eco*RV and *Bgl*II as probe. The results of these hybridisations are shown in figure 3B. The results of the hybridisation of the control strain HP816NI^r show the presence of a restriction target *Xho*I placed at approximately 20 kb of that found within operon *apx*I. The analysis of the recombinant with the insertion of plasmid pApxIΔH2, shows the appearance of two new bands of 1.1 and 4.3 kb and a slight increase of approximately 1kb of the preexisting band of 20 kb. The size of the new bands and the increase of the preexisting one , are the expected from the insertion of the hybrid plasmid pApxΔH2 in the 5′ flanking region of the coding fragment of the second transmembrane helix of the *apx*IA gene of the App genome (Figure 3A, outline 2). The analysis of the recombinant with the plasmid resolved from the second recombination in the same 5′ region where the first took place, show the disappearance of the two bands of lesser molecular mass and a slight decrease of the mobility of the previous 21 kb band which now appears at the same level as the parent strain. This, together with the fact hat the haemolytic activity is identical to that exhibited by the parent strain HP816NI^r, suggests that no additional modifications are introduced in the App genome during all this process (Figure 3, A, and C).

Finally, the analysis of the recombinant with the plasmid resolved from a second recombination in the 3′ flanking region of the segment that codes the second transmembrane helix shows the disappearance of the 4.3 kb band, the maintenance of the 1.1 kb band which was already observed in the recombinant by insertion and a slight decrease of the 20 kb band. This bands distribution is the expected one, due to the disappearance of the coding segment of the second transmembrane helix and its substitution by a *Xho*I target. This new target, inserted in the App genome, gives rise to the 1.1 kb fragment and the consecutive decrease of 1.1 kb in the 20kb band which is observed in the strain 816 Nl^r (Figure 3A and B). Note in CA the presence of large haemolytic halos surrounding the colonies of cultures 1 and 3 and of small haemolytic halos surrounding the colonies of cultures 2, 4 and 5. See also the absence of growth in TS of the cultures 1, 3 , 4 and 5. This recombinant shows a very reduced haemolytic activity as compared to the parent strain HP816 Nl^r and the same haemolytic activity than a serotype 7 App which possesses only the ApxII haemolysin (Figure 3C). This result indicates that the deletion in the second transmembrane helix eliminates or reduces considerably the haemolytic activity of the App ApxIA.. The App modified by the described deletion will be named , from now on, ApxIAH2$^-$.

The recombinant so obtained, characterized by having a deletion in nucleotides 885 to 944 in the *apx*IA gene which code the second transmembrane domain of the *Apx*I exotoxin, has been named AppApxIH2$^-$. On the 10th January 2002 this has been deposited in the Colección Española de Cultivos Tipo with the registration number CECT 5985, according to the conditions established in the Budapest treaty.

E.2.- Analysis of the apx/IIH2$^-$ recombinants.

The genomic DNA samples corresponding to the recombinant by insertion HP816R2 and to the second recombinants obtained from the plasmid pApxIIΔH2$^-$ were digested with the restriction enzyme *Eco*RI. These digestions together with another one performed from a DNA extracted from a culture of the strain HP816Nl^r , were analysed using the Southern-blot. As a

probe, the two DNA fragments amplified by PCR were used. These corresponded to the 5′and 3′ flanking regions of the DNA segment that codes for the second transmembrane helix of the ApxII (Section B). The results of these hybridisations are shown in figure 4B. The results of the hybridisation of the control strain HP816 Nl^r show the presence of two *Eco*RI restriction targets distant from one another 15.7 kb, which delimit a fragment in which operon *apx*II is enclosed. The analysis of the recombinant by insertion of plasmid pApxIIΔH2 shows the disappearance of the 15.7 kb band and the appearance of 3 new bands of 8.2, 7.5, and 0.9 kb. The size of the new bands is the expected from the insertion of the hybrid plasmid pApxIIΔH2 in the 3′ flanking region of the coding segment of the second transmembrane helix of gene *apx*IIA of the App genome (Figure 4A). The analysis of the recombinant with the resolved plasmid from the second recombination in the same 3′ region where it took place the first one, shows the reappearance of a single 15.7 kb band which coincides with that shown by the control strain (Figure 4B). The haemolytic activity is identical to the one shown by the AppApxIH2$^-$ parent strain (Figure 4C). Finally, the analysis of the recombinant with the plasmid resolved from a second recombination by the 3′ flanking region of the segment that codes the second transmembrane helix shows the disappearance of the 13.5 and 0.9 kb bands and the appearance of a new 7.5 kb fragment (Figure 4B). This band distribution is the expected one, from the disappearance of the coding segment of the second transmembrane helix and its substitution by an *Eco*RI target (Figure 4A). This new target inserted in the App genome causes the 15.7 kb *Eco*RI, fragment, which included operon *apx*II in the parent strain, to split in two 8.2 and 7.5 kb fragments (Fig 4A and B). This recombinant is virtually non-haemolytic (Figure 4C). This result indicates that the deletion in the second transmembrane wipes out or reduces in its practical entirety, the haemolytic activity of the App ApxIIA. The ApxIIA modified by carrying out the described deletion will be renamed from now on as ApxIIAH2$^-$. Notice in CA the presence of small haemolytic halos surrounding the colonies of cultures 1 and 3 and the absence of haemolytic halos surrounding the colonies of cultures 2 and 4. Notice also the absence of growth of cultures 1, 3 and 4

in TS.

The recombinant strain so obtained has been renamed AppApxI/IIH2⁻. This is characterized by having a deletion in nucleotides 885 to 944 in the *Apx*IA gene which code a second transmembrane domain of the ApxI exotoxin and furthermore a deletion of nucleotides 885 to 944 of the *apx*IIA gene which codes the second transmembrane domain of the *Apx*II exotoxin. This strain has been deposited in the Coleccion Española de Cultivos Tipo, on the 12th June 2002 with the registration number CECT 5994 as specified in the conditions of the Budapest Treaty on patents.

F.- Analysis of the production of the ApxIAH2⁻ and ApxIIAH2⁻ by the recombinants strains obtained.

To determine if the obtained recombinant strains were still producing the ApxH2⁻ the concentration of the same in the LB medium was determined. The Apx produced were detected using monoclonal antibodies specific to the Apx I and Apx II using immunoassays and Western-blot. As shown in figure 5 (A and B) the production and excretion to the medium of the ApxIAH2⁻ and ApxIIAH2⁻ by the recombinant strains follows the same temporary pattern than the non-modified Apx from the parent wild type strain HP816Nl^r . All haemolysins (modified or not) appear in the culture medium at about the second half of the exponential growth phase and reach the maximum concentration at the beginning of the stationary phase. From this moment onward, the concentration of all haemolysin remains stable or decreases slightly. As observed in the same figure, ApxIAH2⁻ and ApxIIAH2⁻ accumulate until reaching levels similar to those shown in the respective non-modified Apx produced by the wild type parent strain HP816Nl^r . On the other hand, the introduced deletion is very small (18 amino-acids) and only a decrease of 2 kD in the molecular mass of the two ApxH2⁻ is expected. Bearing in mind that the 2 wild type haemolysins have an apparent molecular mass of approximately 105 kDa, a decrease of 2 kDa in its molecular mass is unnoticeable in the polyacrilamide gels and its corresponding Western-blots (figure 6). Finally, we must highlight that in

this same figure do not appear, truncated or improperly processed polypeptidic products. Notice that the 105 kDa in track 3 appears detected only in (C). All these data indicate that the small deletion introduced in both Apx does not hinder that these be synthesized in a full way and exported to the culture medium. Once these have been freed in the culture medium, the ApxH2$^-$ exhibit a stability similar to that shown by the respective non-modified Apx.

G.- Effectiveness of the attenuation of the obtained strains.

To test the degree of attenuation of the two constructed recombinant strains, three months old, male and female LWxLD hybrid swine, were used. Four replicates of swine were used in the different trials. Each one of the strains was administered at a dose of 10^8 cfu in 5 mL of PBS, to each one of the animals of the 3 first groups, by intratracheal injection. Previously this dose was determined as the LD50 for the wild type strain HP816Nl^r in swine of this age. The animals of the fourth group were inoculated only with one dose of 5 mL of PBS. The clinical signs were noted down daily during the 7 days period that lasted the trial. The results are shown in table 2:

Table 2

Strain	Number of Animals	Dosage App (cfu)	Mortality	Days with behaviour disturbances			Days with clinical respiratory signs (b)		
				0-2	3-4	5-6	0-2	3-4	5-6
HP816Nl^r	5	10^8	2/5	5/5	3/3	3/3	5/5	1/3	0/3
AppApxIH2$^-$	10	10^8	0	10/10	9/10	4/10	5/10	3/10	3/10
AppApxI/IIH2$^-$	10	10^8	0	4/10	0/10	0/10	2/10	0/10	0/10
Control (PBS)	5	N.A.	0	0/5	0/5	0/5	0/5	0/5	0/5

(a) Animals with impaired alert behaviour and ability to respond in the presence of the caretaker (Affected/total)

(b) Animals with disturbed respiratory rhythm and/or dyspnea (Affected/ Totals).

Seven days after the inoculation the animals were sacrificed and the observed macroscopic lesions, in the respiratory organs, were recorded. Bacteriological examinations were also carried out at the necropsy.

The results obtained in this trial are summarized in Table 3

Table 3

Strain	Number of animals	Dosage App (cfu)	Mortality	Animals with lung lesions	Mean lung lesion Index	Animals from which App was isolated
HP816NI^r	5	10^8	2/5	4	11.6± 2.1	3
AppApxIH2$^-$	10	10^8	0	7	3.2 ± 4.6	3
AppApxI/IIH2$^-$	10	10^8	0	0	0	8
Control (PBS)	5	N.A.	0	0	0	0

Two out of 5 animals of this group died during this period of time. At the necropsy, four out of five animals, showed severe lung lesions. The animals which had been inoculated with the strain AppApxIH2$^-$ showed also a modification of their behaviour, although these signs slowed down from the forth inoculation day. The clinical signs were milder and were only observed in 50% of the pigs. Although none of the animals of this group died during the trial, 70 % of them showed lesions at the necropsy although all of them were found to be milder than the previous group. The third group was inoculated with the strain AppApx/IIH2$^-$. Although four of the animals showed mild modified behaviour, these slowed down from the 48th hour post-inoculation. The two animals that showed limited clinical signs also recovered

within 48 hours after the inoculation. No lung lesions were observed in none of the animals at the necropsy. The assessment of the lung lesions was done according to Hannan et al; ((Research in Veterinary Science 33:76-88 (1982)). The values shown are the arithmetical means of each group together with the standard deviation. According to these results, the AppApxI/IIH2$^-$ strain is non-virulent and can be used safely as a live vaccine. It is important to highlight that the App strain inoculated was recovered in 80% of the pigs of this group, seven days after its administration.. This result indicates that the viability of the AppApxI/IIH2$^-$ strain in an experimental infection is not modified in spite of the fact that it is devoid of haemolytic activity. This fact is important if we bear in mind that it is essential that the microorganism remains viable so that the Apx exotoxins can be generated and freed. Without the production of the Apx exotoxins, the attenuated strain could not be used as live vaccine since this would be unable to induce an immune response which would protect the animal against future infections (Reimer et al; Microbial Pathogenesis 18:197-209 (1995). In all trials a strong immunogenic response has been achieved.